Liposomal Drug Formulation

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EXECUTIVE SUMMARY

Wound care is an important aspect of post-operative surgery, and can play an integral role in wound healing. The bactericidal activity of the dressing will not only depend on the type and amount of drug loaded in the material but also on the form in which the drug was loaded. The first objective of the current project was to investigate whether the bactericidal activity of the DRDC-Avitar dressing would be prolonged by loading the dressing with a liposome-encapsulated drug. Liposomes are non-immunogenic, biodegradable and biocompatible lipid vesicles that have been increasingly explored as novel drug delivery systems. In this project various liposomal formulations were evaluated. This study revealed that the antibacterial activity of liposomal drug-loaded dressings was prolonged, compared to that of free drug-loaded material.

Another objective was to evaluate the efficacy of the DRDC dressing to deliver the bactericidal drug to the wound by determining kinetics of drug elimination from the treated tissue, as well as the amount of drug remaining in the material after its application to the wound should be determined. Since only small tissue samples are obtained from *in vivo* experiments, emphasis was put on developing a drug extraction method that would yield high drug recovery from tested tissues and dressings. The validity of the method was confirmed using muscle samples obtained in a previous DRDC experiment.

SOMMAIRE

Les soins de la plaie constituent un aspect important de la chirurgie postopératoire et peuvent jouer un rôle intégral dans la cicatrisation de la plaie. L'activité bactéricide du pansement ne dépendra pas seulement du type ou de la quantité de médicament incorporé dans le matériel mais aussi de la façon dont le médicament a été incorporé. Le présent projet visait en premier lieu à déterminer si le pansement mis au point par RDDC et Avitar aurait une activité bactéricide plus longue si le médicament incorporé était encapsulé dans des liposomes. Les liposomes sont des vésicules lipides non immunogènes biodégradables et biocompatibles qu'on étudie de plus en plus à fond comme nouveaux modes d'administration. Nous avons évalué diverses formulations liposomales dans le cadre de ce projet. Les résultats ont révélé que l'activité antibactérienne des pansements à médicament encapsulé dans des liposomes durait plus longtemps que celle des pansements où le médicament incorporé était libre.

Un autre objectif du projet était d'évaluer l'efficacité du pansement de RDDC à diffuser le médicament bactéricide dans la plaie, en déterminant la cinétique de l'élimination du médicament du tissu traité ainsi que la quantité de médicament qui reste dans le matériel après application de celui-ci sur la plaie. Toutefois, comme les expériences *in vivo* n'ont donné que de petits échantillons de tissu, nous avons porté notre effort sur l'élaboration d'une méthode d'extraction de médicament qui permettrait de récupérer une bonne quantité de médicament des tissus traités et des pansements. La validité de la méthode a été confirmée sur des échantillons de muscles obtenus d'une expérience effectuée antérieurement par RDDC.

ABSTRACT

The ability of wound dressings to prevent undesired growth of microorganisms when applied to a wound surface is a critical factor in choosing a wound-care strategy. The bactericidal activity of the dressing will not only depend on the type and amount of drug loaded in the material but also on the form in which the drug was loaded. One objective of the current project was to investigate whether the bactericidal activity of the DRDC-Avitar dressing would be prolonged by loading the material with a liposome-encapsulated drug. Various liposomal formulations were tested and the pharmacokinetic profile of each formulation was evaluated. This study revealed that the antibacterial activity of liposomal drug-loaded dressings was prolonged, compared to that of free drug loaded material. However, more extensive studies are required to assess the feasibility of liposome utilisation in wound dressings.

To evaluate the ability of the DRDC-Avitar dressing to deliver the bactericidal drug to the wound, kinetics of drug elimination from the treated tissue as well as the amount of drug remaining in the dressing material after its application to wound must be determined. Since only small tissue samples are obtained from *in vivo* experiments, the second objective of the project was to develop a drug extraction method that would yield high drug recovery from tested tissues and dressings. The developed procedure has a high recovery rate (>90%) and is easy to perform. The extracted samples may used for HPLC as well as for LC-MS analysis. The validity of HPLC method was confirmed using muscle samples obtained in a previous DRDC experiment.

RESUME

Quand on soigne une plaie, il est essentiel de choisir un pansement qui empêche des microorganismes indésirables de se multiplier à la surface de la plaie. L'activité bactéricide du pansement ne dépendra pas seulement du type ou de la quantité de médicament incorporé dans le matériel mais aussi de la façon dont le médicament a été incorporé. Le présent projet visait en premier lieu à déterminer si le pansement mis au point par RDDC et Avitar aurait une activité bactéricide plus longue si le médicament incorporé était encapsulé dans des liposomes. Nous avons évalué diverses formulations liposomales dans le cadre de ce projet et nous avons également testé le profil pharmacocinétique de chaque formulation. Les résultats ont révélé que l'activité antibactérienne des pansements à médicament encapsulé dans des liposomes durait plus longtemps que celle des pansements où le médicament incorporé était libre. Des études plus approfondies sont toutefois nécessaires pour juger s'il est faisable d'utiliser les liposomes dans les pansements.

Pour évaluer la capacité du pansement RDDC-Avitar à diffuser le médicament bactéricide dans la plaie, il faut déterminer la cinétique de l'élimination de médicament du tissu traité et la quantité de médicament resté dans le matériel de pansement après l'application de celui-ci. Puisque les expériences in vivo n'ont donné que de petits échantillons de tissus, le second objectif du projet consistait à élaborer une méthode d'extraction de médicament qui permettrait de récupérer des tissus traités et des pansements une bonne quantité de médicament. La procédure, très facile à appliquer,

donne un taux élevé de récupération (> 90 %). On peut utiliser les échantillons extraits pour l'analyse par CLHP et par CL/SM.

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1.0 Introduction

Efficient and prompt wound care plays a crucial role in the successful healing of wounds. Different types of bactericidal wound dressings are commercially available. They differ in their drug composition, absorbance capacity, suggested time of application, etc. DRDC Toronto has designed a wound dressing that is minimally adherent, highly absorbent and can be used as a drug delivery system. A first study was designed to investigate whether the antibacterial properties of the DRDC-Avitar wound dressing could be prolonged by encapsulating the bactericidal agent into liposomes. Liposomes are safe, biodegradable, non-immunogenic phospholipid vesicles that could be used as drug carriers (1). Lipophilic as well as hydrophilic drugs have been efficiently encapsulated into liposomes (2). Drug encapsulation into liposomes increases its biologic half-life, and improves its therapeutic activity (1). Recently, several liposomal formulations have been approved in USA, Europe and Japan for clinical use (3). Development of liposomal antibacterial-drug formulation for incorporation into wound dressings was considered to unite the antibacterial activity with excellent tolerability, lack of immunogenicity and sustained release of liposomes. Among the potential candidates for encapsulation into liposomes, mafenide, a hydrophilic, broad-spectrum antibacterial drug, was chosen because it possesses excellent bactericidal properties against *Ps. aeruginosa* and *Staph. epidermidis,* the predominant microorganisms recovered in wounds.

DRDC Toronto had also a requirement for the development of an extraction procedure for the recovery and quantification of chlorhexidine from pig tissue samples. The sample preparation process has a direct impact on accuracy, precision, and quantification limits, and is often the rate-determining step for many analytical methods. Tissue samples usually must be processed in order to isolate and concentrate organic analytes from the sample matrix and provide a suitable sample extract for instrumental analysis. Although the significance of the sample preparation is often overlooked, it is arguably the most important step in the analytical process. The present extraction method was developed using muscle tissue samples taken from pig wounds treated with chlorhexidine-loaded DRDC wound dressings. There are several procedures for chlorhexidine extraction from tissues (4-6). Most of them use solid phase extraction for sample purification. Though this type of extraction gives highly purified samples, significant loss of analyte may be associated with this method (7). These methods could not be used since only small tissue samples (< 50 mg) are available for the analysis. The second objective of this research contract was therefore to develop and demonstrate the potential of the new extraction method to increase the analyte recovery, so it would be measurable by HPLC. However, despite the improvements in the extraction procedure, the sensitivity of HPLC may still be insufficient for accurate determination of low concentrations of chlorhexidine in the samples. The extraction method developed was therefore further modified to suit both HPLC and (a more sensitive) LC-MS analysis.

Finally, the developed extraction method was used to analyse pig muscle samples obtained from previous *in vivo* experiments. Promising results have been obtained from selected extracted samples analyzed by HPLC.

2.0 STUDY 1: LIPOSOMAL MAFENIDE FORMULATION

2.1 Objective

The objective of the study was to develop a liposomal formulation of the antibacterial drug mafenide acetate for incorporation into the DRDC-Avitar wound dressing.

2.2 Methods and results

2.2.1 Spectrophotometric analysis of mafenide acetate

This is a simple quantitative method of drug analysis. The main drawbacks of the method are its relatively low sensitivity and the large sample volume needed for the analysis. In this project, this method was used for quantification of mafenide encapsulation into liposomes. The method was as follows:

a) Prepare 10 mM mafenide acetate (4-Aminomethylbenzenesulfonamide acetate salt; Sigma-Aldrich Canada, Ltd, Cat. No. A3305) stock solution in double distilled water and store it at 4°C for up to 1 week.

b) On the day of the experiment prepare a calibration curve for mafenide acetate (0.1-1 mM) by diluting the stock solution with 0.01N HCl. Prepare each concentration point in triplicates.

c) Measure the UV absorbance of mafenide acetate at 267 nm immediately after sample preparation.

d) To measure an unknown mafenide acetate sample (aqueous solution of mafenide), dilute it 1:1 with 0.02N HCl to achieve 0.01N HCl in the final sample.

e) Measure the sample absorbance immediately after the dilution.

f) Calculate mafenide acetate concentration in the unknown sample using the prepared mafenide acetate calibration curve, and multiply the concentration value obtained by 2.

Fig. 1 depicts a typical mafenide acetate calibration curve obtained when following the procedure outlined above. All mafenide acetate measurements in this report were performed based on this assay.

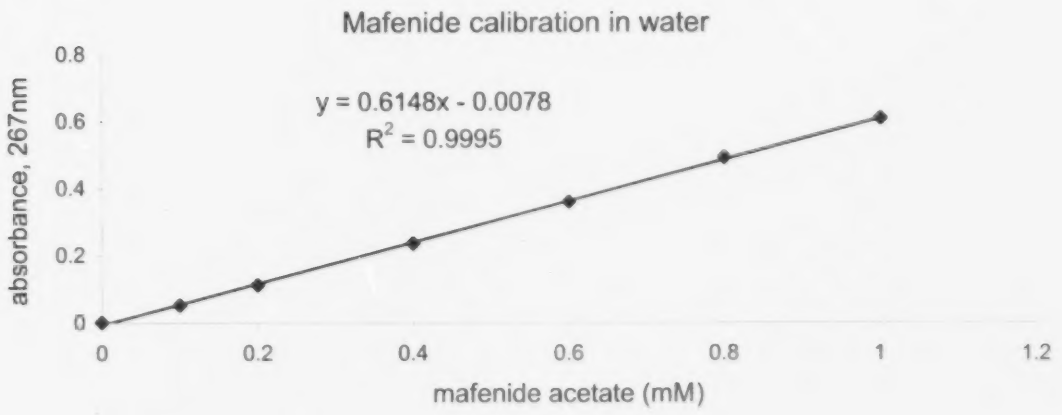


Figure 1. Mafenide acetate absorbance in water.

2.2.2 Mafenide acetate encapsulation into liposomes

Mafenide acetate is a broad-spectrum antibacterial drug that belongs to sulfa family. Since it is water soluble, it can be loaded into the intraliposomal aqueous phase. Liposomes are artificial lipid vesicles that represent a promising carrier for targeted delivery of drugs or other biologically active molecules (8). The size of liposomes as well as the number of lamellae per vesicle can be controlled within certain limits. The lipid composition of liposomal membrane profoundly influences their biological behaviour and kinetics of drug release from liposomes (9). Therefore, three lipid compositions were tested in this study.

2.2.2.1 COMPOSITION OF LIPOSOMAL MEMBRANE

a) 1,2-Dipalmitoyl-sn-Glycero-3-phosphocholine (DPPC) – 100%

b) DPPC/Cholesterol – 8/2 mole ratio

c) DPPC/Cholesterol – 7/3 mole ratio

2.2.2.2 Preparation of Multilamellar Vesicles (MLV) loaded with mafenide acetate

a) Prepare mafenide acetate solution in the concentration range of 10-20%w/v or 0.4-0.8M – this is a re-hydration solution.

b) Dissolve the lipid(s) in pure tert-butanol (for each 100 mg of lipid(s) add 3-5 ml of tert-butanol). Sometimes, gentle warming to 40-50°C is needed.

c) Freeze-dry tert-butanol solution overnight to obtain thin dry lipid film;

d) Re-hydrate the dry film with the prepared mafenide solution to achieve a 10% w/v lipid concentration. During re-hydration process MLV are formed.

e) Store the resulted MLV at 4°C.

2.2.2.3 DOWNSIZING OF MAFENIDE-LOADED LIPOSOMES

The following steps will downsize MLV to Large Unilamillar Vesicles (LUV).

a) Preheat MLV loaded with mafenide acetate to the phase-transition temperature of the core lipid, i.e. 53°C.

b) For a small-scale preparation (<2 ml), use Avanti Mini-extruder (Avanti Polar Lipid, Inc. Alabaster, Alabama, US).

    a. Preheat the extruder to 60°C;

    b. Push preheated MLV 19 times through 400 nm-pore-size polycarbonate membrane to achieve LUV of ~400 nm.

c) For a large-scale preparation (2-100 ml), use High-Pressure Extrusion Device (Lipex Biomembrane, Vancouver, BC, Canada:

    a. Preheat the Extrusion Device to 60°C;

d) Run preheated MLV through the device 10 times, using 400 nm-pore-size double-stacked polycarbonate membranes.

e) Measure size distribution of resulted LUV by Photon Correlation Spectroscopy using Coulter sub-micron particle analyzer.

Size distribution of 400±50 nm was obtained for both, small and large scale downsizing methods.

2.2.3 CHARACTERIZATION OF LIPOSOMAL MAFENIDE PREPARATION

2.2.3.1 DETERMINATION OF EFFICACY OF MAFENIDE ENCAPSULATION

The efficacy of mafenide acetate encapsulation was assessed in LUV preparations with three different membrane-lipid compositions as detailed below:

a) Separate the supernatant from the liposomal pellet using ultracentrifuge (12000 rpm, room temperature, 10 min).
b) Collect the supernatant in a separate test-tube.
c) Re-suspend the liposomal pellet with cold saline followed by another supernatant separation, to eliminate residues of free, non-loaded mafenide acetate.
d) Dissolve liposomes with 1% SDS detergent to release loaded mafenide acetate.
e) Measure mafenide acetate absorbance at 267 nm after its release from LUV (ABS_{LUV}).
f) Measure mafenide acetate absorbance of previously collected supernatant (ABS_{SUP}).

g) The percentage of mafenide acetate encapsulation was calculated as: $[ABS_{LUV}/(ABS_{LUV}+ ABS_{SUP})] \times 100$.

The intra-liposomal aqueous volume of LUV was ~ 15-22% of the total volume of liposomal formulation. Percentage of mafenide acetate encapsulation for the three tested liposomal formulations, calculated as described above, was close to this number (20±2%). These results suggest that the mafenide acetate concentration inside the liposomes (MAF_{LUV}) was identical to that in the extraliposomal aqueous phase (MAF_{SUP}). This finding suggests that the mafenide acetate does not leak from any of the three liposomal formulations during the loading and washing process. At this point, no preference could be made among the formulations.

2.2.3.2 Determination of stability of liposomal mafenide acetate formulations – release kinetics of mafenide acetate

The stability of three liposomal mafenideacetate formulations was assessed as follows:

a) Separate the supernatant from the liposomal pellet using ultracentrifuge (12000 rpm, room temperature, 10 min).
b) Collect the supernatant in a separate test-tube.

c) Re-suspend the liposomal pellet with cold saline followed by another supernatant separation, to eliminate residues of free, non-loaded mafenide acetate.

d) Add re-suspended (washed) liposomes to mediums containing 0% and 1% human plasma (15µl of liposomes to 1.5 ml of plasma or PBS).

e) Incubate the samples for 0.5, 1, 2, 3, 4, and 24 h at 37°C.

f) Collect the supernatant at pre-determined time-points after centrifugation (12000 rpm, room temperature, 10 min).

g) Measure mafenide acetate absorbance at 267 nm in the collected supernatant samples.

h) Calculate mafenidacetate concentration in the supernatant samples based on mafenide acetate calibration curves in 0% and 1% human plasma. This concentration represents mafenide acetate that has leaked out of liposomes since the start of the incubation until the measured time point.

i) Plot leakage kinetics of mafenide acetate as a function of time.

Fig. 2 depicts the leakage kinetics of mafenide acetate from 3 different liposomal formulations. The results suggest that the liposomal preparation based on lipid composition of DPPC/Cholesterol (7/3 mole ratio) is less susceptible to leakage and therefore is capable of preserving mafenide acetate inside the liposomes for the longest period of time. Since the purpose of this study was to achieve a stable mafenide acetate

encapsulation, the formulation of DPPC/Cholesterol (7/3 mole ratio) was chosen to be the formulation of choice for this study.

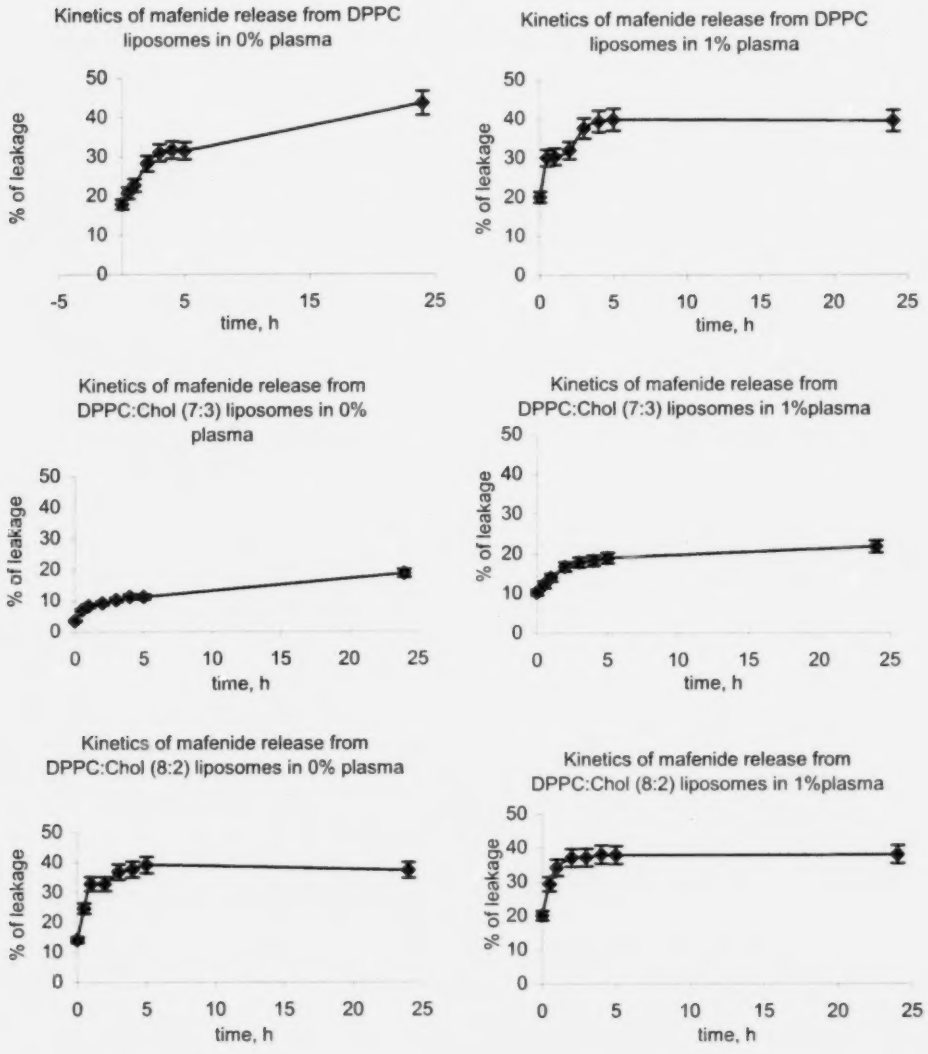

Kinetics of mafenide release from DPPC liposomes in 0% plasma
% of leakage
time, h
Kinetics of mafenide release from DPPC liposomes in 1% plasma
% of leakage
time, h
Kinetics of mafenide release from DPPC:Chol (7:3) liposomes in 0% plasma
% of leakage
time, h
Kinetics of mafenide release from DPPC:Chol (7:3) liposomes in 1%plasma
% of leakage
time, h
Kinetics of mafenide release from DPPC:Chol (8:2) liposomes in 0% plasma
% of leakage
time, h
Kinetics of mafenide release from DPPC:Chol (8:2) liposomes in 1%plasma
% of leakage
time, h

Figure 2. Leakage kinetics of mafenide acetate from 3 different liposomal formulations in 0% plasma and 1% plasma medium

Presence of 1% plasma de-stabilized the liposomes during the onset of the incubation, but did not influence the total percent of leakage at the end of the incubation. Leakage of 20% was detected in the formulation of choice after 25 hours of incubation at 37°C. Fastest leakage occurred during the first 5 hours of the incubation, then liposomal membrane stabilized, and leakage rate slowed down and remained constant until the end of the experiment 25 hours later.

2.2.3.3 Comparison of *in vitro* bactericidal activity of liposomal and free mafenide acetate

The *in vitro* bactericidal activity of liposomal and free mafenide acetate-loaded wound dressings against Gram-negative and Gram-positive bacteria was determined. Four different dressings were tested:

1. NuGauze®: Non-woven rayon and polyester sponges that provide improved strength, absorbency, and scrubbing texture.
2. Allevyn®: Hydrocellular dressing combines an absorbent hydrocellular pad sandwiched between a perforated non-adherent wound contact layer and a waterproof outer film. Absorbs up to five times more fluid than hydrocolloids. Outer polyurethane film is waterproof but vapour-permeable, so it allows excess moisture to evaporate.
3. HydraSorb®: Hydrophilic, polyurethane foam dressing is a soft, non-linting, pre-cut pads that cushion and protect the wound site. They are

semi-occlusive, allowing for the transfer of oxygen. Absorbs exudates and provides a moist wound environment.

4. SuperSoft®: Hydrophilic, polyurethane foam dressing with SuperSoft® particles. Chemical and physical characteristics close to those of HydraSorb®.

Experimental procedures were as follows:

a. Prepare 5% free mafenide solution and 5% liposomal mafenide preparation (DPPC 100%).
b. Prepare the desired amount of dry dressings (1cmx1cm).
c. Soak the dressings in either 5% free mafenide acetate solution or 5% liposomal mafenide acetate preparation for 30 min.
d. Express from each dressing the excess drug solution.
e. Seed Mueller-Hinton Agar plates with 10^7 CFU/ml of *Ps. aeruginosa* or *Staph. epidermidis* and let stand until the inoculum dried on the surface of the agar.
f. Place each expressed dressing in the centre of each plate, and incubate at 37°C for 24 hours.
g. Corrected zone of inhibition (CZOI) is measured by determining the area with no growth around the dressing less the area of the dressing itself.

h. Each dressing was then transferred to a freshly seeded Mueller-Hinton agar plate and incubated for another 24 hours.

i. The dressings were transferred daily and the CZOI measured until no zones of inhibition were visible.

Figures 3 and 4 indicate that there is a noticeable difference in bactericidal efficacy of free and liposomal mafenide acetate-loaded dressings. Loading into liposomes has preserved bactericidal activity of mafenide acetate for at least one day more, compared to free mafenide acetate. The results also indicate that free as well as liposomal mafenide acetate loaded into HydraSorb® and SuperSoft® dressings resulted in broader zone of inhibition than the same preparations loaded into Allevyn® and NuGauze® dressings (Fig. 3). This finding suggests that HydraSorb® and SuperSoft® dressings are more effective in active drug release than Allevyn® and NuGauze®. Results obtained from *Ps. aeruginosa* (Fig.4) indicate that dressings loaded with liposomal mafenide acetate showed higher bactericidal activity than dressings loaded with free mafenide acetate during 4 days of the 7-day experiment. The interesting finding in this experiment was that Nu Gauze dressing loaded with liposomal mafenide acetate preserved its bactericidal activity at the same level for 4 days, suggesting that rate of liposomal mafenide acetate release from this dressing remains constant for at least 4 days.

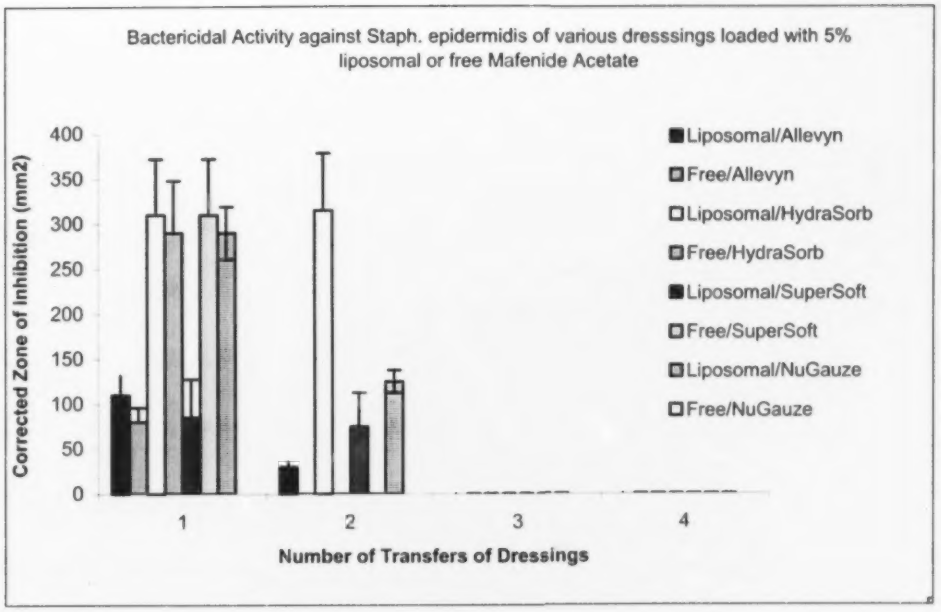


Figure 3. *In vitro* bactericidal activity of free and liposomal mafenide acetate-loaded wound dressings against Gram-positive bacteria.

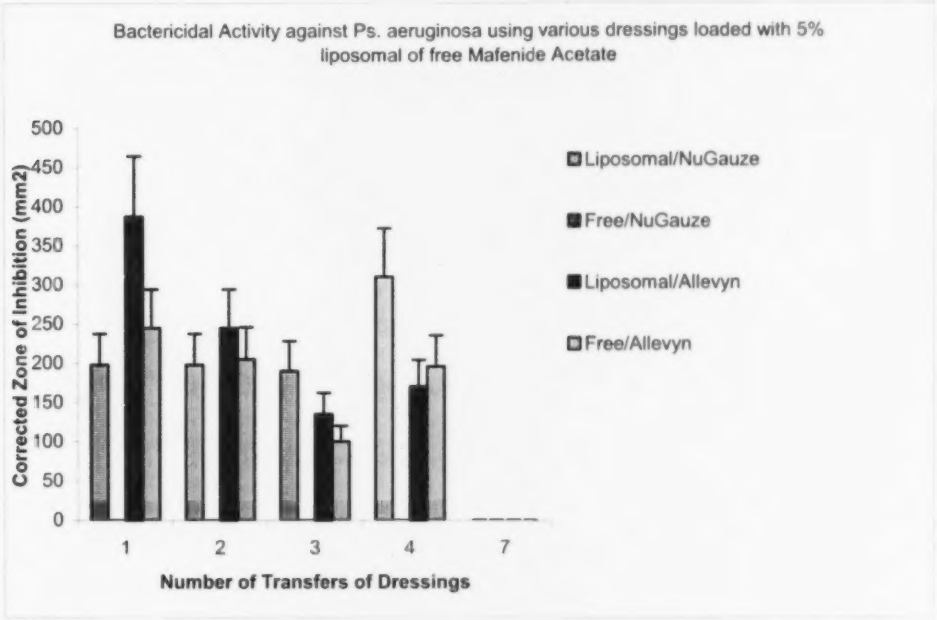


Figure 4. *In vitro* bactericidal activity of free and liposomal mafenide acetate-loaded wound dressings against Gram-negative bacteria.

2.3 Closing Remarks

Loading biocompatible, biodegradable and non-immunogenic liposomes with an antibacterial agent such as cefoxitin had been previously demonstrated (10). This study shows that the antibacterial drug mafenide acetate can be encapsulated into liposomes with reasonable loading efficacy. The mafenide concentration in liposomes was identical to that in the re-hydration solution, and could be as high as 20%w/v. Mafenide acetate loading into liposomes resulted in its delayed release and prolonged antibacterial activity *in vitro*. The prolongation of antibacterial activity of mafenide acetate by loading it into liposomes did not interfere with the extent of its antibacterial activity. Since prolongation of the antibacterial activity of a wound dressing may mean less frequent wound dressing changes for patient and thus, easier management of wounds for medical staff, future investigation of this topic is encouraged.

In addition, this study has revealed that the extent of antibacterial activity depends not only on the form of the drug loaded into a wound dressing, but also on a type of wound dressing used. It was determined that HydraSorb® and SuperSoft® dressings released higher amounts of mafenide acetate than Allevyn® and NuGauze® dressings, thus providing a better antibacterial protection for the same mafenide acetate preparation.

3.0 STUDY 2: METHOD DEVELOPMENT OF CHLORHEXIDINE EXTRACTION FROM TISSUE SAMPLES

3.1 Objectives

The main objective of this study was to develop a method for chlorhexidine extraction from muscle tissues. The specific objectives were:

1) to perform extensive literature search on chlorhexidine tissue extractions;
2) to modify existing methods, so they will suit the experimental design;
3) to develop the detailed extraction procedure for chlorhexidine that would allow a high percentage of drug recovery;
4) to combine the last step of the extraction procedure with HPLC analysis of samples;
5) to determine the minimal chlorhexidine concentration in dressings, which enables accurate HPLC analysis of chlorhexidine in tissues treated with those dressings.

3.2 Methods and Results

Anxtensive review of the literature revealed that the vast majority of assays for chlorhexidine extraction from tissue samples have used a solid extraction method (4-6). This extraction method on one hand eliminates many impurities from tissue samples, but on the other hand decreases the percentage of chlorhexidine recovery (7). Since the current procedure was developed for assaying small weight tissue samples, the component of solid extraction was removed from the developed procedure, and extensive

filtering was used instead, in order to decrease sample loss. Detailed protocol of the modified extraction procedure is presented below.

3.2.1 Chlorhexidine extraction method

a) Prepare 5 chlorhexidine solutions of different concentrations, ranging from 1 to 100 μg/ml in water/acetonitrile (65%/35% v/v)).

b) Take 500 μl of each solution and dissolve aliquot in 5 ml of methanol.

c) Evaporate the samples to dryness in a stream of nitrogen with gentle heating (60-70°C in a hood for ~1 h).

d) Reconstitute the residue in 500 μl of diluting solvent (65% water/35% acetonitrile) using a Vortex mixer.

e) Draw each sample (~500 μl) using a small needle, and filter it through an HPLC syringe-filter (45 μ) directly into an HPLC vial. These samples are referred to as "with extraction" samples.

f) Filter a small aliquot of each solution prepared in step (a) through HPLC syringe-filter (45μ) into HPLC vials. These samples are referred to as "without extraction".

g) Run the filtered samples through HPLC:

   i. Column: Zorbax SB-C8, 4.6 mm x 150mm, 5μ;

   ii. Mobile phase: 0.05 M Phosphate buffer, pH 2.5/Acetonitrile (66:34 v/v)

   iii. Flow rate: 1.0 ml/min

   iv. Run time: 12 min

   v. Detector: UV at 245 nm

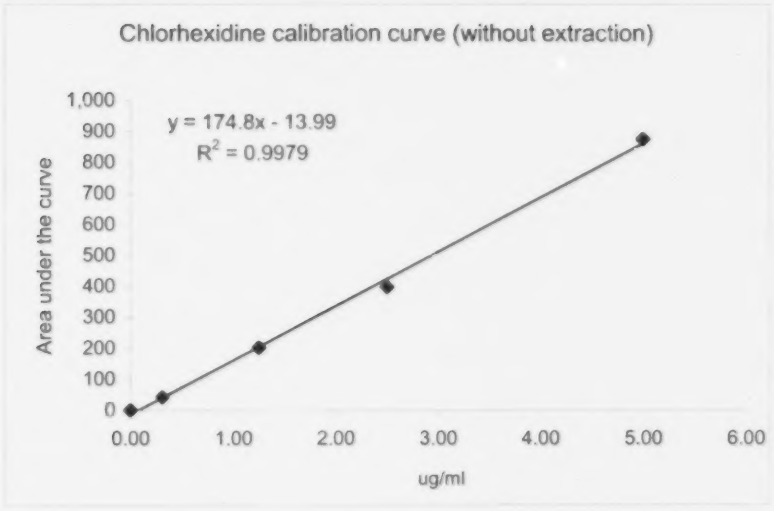
vi. Injection volume: 50 µl

vii. Temperature: 30°C

h) Measure and compare areas of chlorhexidine peaks of both sets of samples (with and without extraction).

i) Calculate percentage of drug recovery by dividing area of extracted samples by area of non-extracted samples and multiplying the result by 100.

As was expected from using an extraction procedure that does not include any gel extraction (such as solid phase extraction), chlorhexidine recovery in the process was close to 100%. Chlorhexidine calibration curves derived from HPLC analysis of extracted/non-extracted samples are presented in Figure 5. Both calibration curves are almost identical, suggesting that the chlorhexidine loss during the extraction process was minimal.



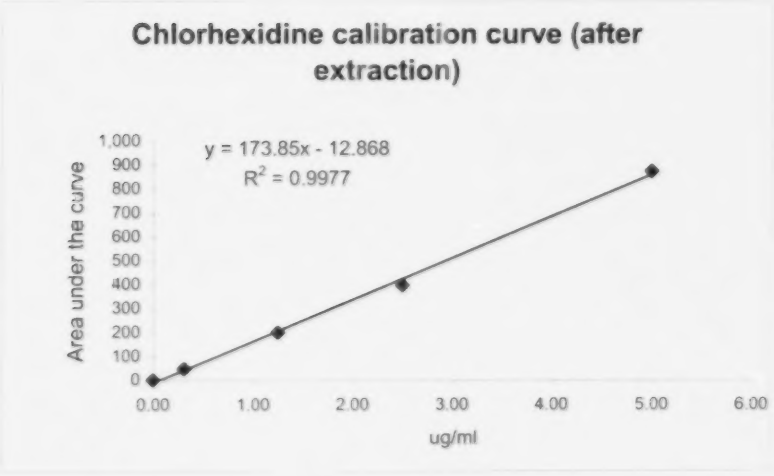


Figure 5. Calibration curves of chlorhexidine without extraction and after extraction method developed.

3.2.2 Chlorhexidine extraction from muscle tissue samples

The chlorhexidine extraction method described above will ultimately be used for assessing the drug content in the wound bed of contaminated full-thickness porcine wounds covered with the DRDC drug-loaded dressings. Since limited experimental samples are available from these *in vivo* experiments, the protocol

detailed below was followed to simulate the chlorhexidine-loading of pork tissues and test the extraction procedures developed in muscle tissue.

a) Prepare 8 dressings (4xHydraSorb and 4xSuperSoft; 1cmx1cm) and weigh them.
b) Soak the dressings in 10% chlorhexidine solution.
c) Express from each dressing the excess drug solution.
d) Weigh each dressing.
e) Prepare slices of sample from pork chops (1 cm$_x$1 cm$_x$0.2 cm).
f) Weigh each slice.
g) Apply one drug-loaded dressing on each pork sample following these steps:
    i. Place a pork sample on a glass plate.
    ii. Apply the dressing on the pork sample.
    iii. Cover the dressing with paraffin film.
    iv. Cover the whole "sandwich" with PVC tape.
h) Put the glass plate with the "sandwich" into incubator set on 30°C for 16h.
i) After the incubation is completed:
    i. Weigh the dressings and freeze for subsequent extraction using the Bodycote chlorhexidine extraction procedure developed for dressing samples.
    ii. Weigh each pork sample.
j) Using a 4 mm punch, take 3-4 samples per piece of tissue covered by the dressing.

k) Weigh each small piece of tissue and put it into a separate glass-tube.

l) Use 20 µg lidocaine as an internal standard, added to each sample.

m) Extract the small tissue samples (~0.035 g) by addition of 5 ml methanol, followed by vigorous vortex shaking and centrifugation (3000 rpm, 10 min, 18°C).

n) Transfer the supernatant to a separate glass test-tube.

o) Evaporate the supernatant to dryness (as described in 3.2.1) in a stream of nitrogen under gentle heating (~1 h).

p) Reconstitute the residue in 500 µl of diluting solvent (65% water/35% acetonitrile) using a Vortex mixer.

q) To precipitate impurities, centrifuge the reconstituted sample in ultracentrifuge (3000 rpm, 10 min, 18°C).

r) Draw the sample using a small needle, and filter it through an HPLC syringe-filter (45µm) directly into an HPLC vial.

s) Run the filtered samples in HPLC (as described in 3.2.1).

The chlorhexidine concentration in the pork samples was calculated as follows:

a) Build a calibration curve of chlorhexidine (based on HPLC-standards; concentration range 1-20 µg/ml) adding 50 µg/ml lidocaine to each sample as an internal standard.

b) Determine areas of chlorhexidine peak and lidocaine peak.

c) Adjust area of chlorhexidine signal, based on area of lidocaine signal (accounts for sample loss during extraction procedure).

d) Using chlorhexidine calibration curve, calculate chlorhexidine concentration in the sample.

e) Knowing the sample volume (500 µl), calculate the total amount of chlorhexidine in the sample.

f) Knowing the tissue weight, calculate chlorhexidine concentration in each sample (in mg/g tissue).

g) Calculate the concentration in the treated pork tissue by making an average of three small tissue samples that came from the same pork sample.

The results obtained are summarized in Tables 1 and 2.

Table 1. Weight of dressings prior to loading with chlorhexidine, after expression of excess drug and after incubation with pork tissues.

Type of dressing	Weight of dry dressing, prior to loading with 10% CH (mg)	Weight of dressing after soaking and expression (mg)	Weight of 10% CH solution in the dressing (mg)	CH amount in the expressed dressing (mg)	Weight of dressing after removal from tissue (mg)	Weight of pork tissue before incubation with dressing (mg)	Weight of pork tissue after incubation with dressing (mg)
1) SuperSoft	142	263	121	12.1	161	799	667
2) SuperSoft	112	229	117	11.7	127	991	730
3) SuperSoft	169	333	167	16.7	285	1317	1188
4) SuperSoft	133	256	123	12.3	188	1172	1055
5) HydraSorb	202	333	131	13.1	291	865	747
6) HydraSorb	218	372	154	15.4	324	1027	913
7) HydraSorb	225	374	149	14.9	296	1147	980
8) HydraSorb	194	354	160	16.0	241	1089	766

There was no significant difference in chlorhexidine concentration between tissues covered with drug-loaded HydraSorb or SuperSoft dressings. This finding

suggests similar efficacy of both dressing in releasing chlorhexidine into the tissues. Average amount of chlorhexidine in the muscle tissues to which the dressing was applied was 3.8 ± 1.55 mg tissue.

Table 2. Chlorhexidine concentration in extracted pork samples covered with wound dressings loaded with 10% chlorhexidine

Type of wound dressing	Tissue Weight (mg ±SEM[1]	RT[2] (Lido[3])	Area (Lido) ±SEM	RT (CH[4])	Area (CH) ±SEM	CH in sample (ug/ml ±SEM)	Amount of CH in sample (ug ±SEM)	CH in tissue (mg/g±SEM)	CH amount in the whole tissue (mg)[5]
1) SuperSoft	39±12	2.14	16261± 254	3.52	35267± 1924	229±12	114±6	2.94±0.16	1.96±0.12
2) SuperSoft	38±7	2.14	16325± 247	3.51	48521± 1625	315±11	157±6	4.13±0.18	3.01±0.11
3) SuperSoft	52±18	2.14	17558± 781	3.34	73175± 2158	476±14	238±7	4.57±0.22	5.43±0.27
4) SuperSoft	37±9	2.15	16543± 289	3.33	73779± 2643	480±17	240±9	6.49±0.27	6.85±0.28
5) HydraSorb	44±15	2.15	15832± 295	3.38	63442± 2151	412±14	206±7	4.69±0.21	3.5±0.15
6) HydraSorb	49±8	2.14	16847± 278	3.45	47144± 1562	306±10	153±5	3.13±0.14	2.86±0.12
7) HydraSorb	53±12	2.15	17201± 485	3.39	68461± 2282	445±15	222±8	4.20±0.25	4.11±0.24
8) HydraSorb	51±17	2.14	16982± 367	3.44	63730± 1875	414±12	207±6	4.07±0.19	3.12±0.12

[1] SEM – Standard Error of the Mean [2] RT – Retention time [3] Lido – lidocaine [4] CH – chlorhexidine [5] – CH amount in the whole tissue was calculated using weight of pork tissues after incubation with dressings (last column in Table 1)

During the study, it was observed that the extraction method, together with providing maximum chlorhexidine recovery, has a disadvantage: its inability to eliminate completely fat droplets present in the samples. Though these droplets

do not interfere with the chlorhexidine analysis, they significantly shorten the lifespan of the HPLC guard column. No efficient method to eliminate fat droplets from the samples was found. The only means to decrease the fat fraction is ultra-centrifugation of reconstituted samples followed by filtering through 0.45μm syringe-filters.

3.2.3 Detection limit of chlorhexidine in muscle tissue samples

Commercially available chlorhexidine-loaded wound dressings typically contain 0.5-1% chlorhexidine. Increased blood flow in the wound bed, taken together with a certain amount of drug left in the dressing mean that the drug tissue levels may be considerably lower than 0.5% in the wound bed. An experiment was therefore designed to determine the minimal concentration of chlorhexidine detectable in tissue sample covered with drug-loaded dressings. The experimental procedures followed were:

a) Prepare 5 chlorhexidine solutions: 0.1, 0.2, 0.4, 0.7, 1.0% w/v in distilled water.
b) Prepare 5 SuperSoft dressings (1cmx1cm) for each chlorhexidine concentration and soak them in chlorhexidine solutions.
c) Follow the procedure 3.2.1 from step (b) through step (h).

In order to determine the range of concentrations that could be accurately measured in tissues following extraction procedure, pork samples were covered

with wound dressings soaked in different chlorhexidine solutions. Five dressings were tested for each concentration, and 3 biopsies were taken from each slice of incubated pork sample. Table 3 shows the average results of this experiment. The minimal detectable chlorhexidine concentration in soaking solution is 0.2% w/v. Incubation with dressings soaked in 0.1% chlorhexidine solution resulted in chlorhexidine signals with very wide standard deviation, in pork samples treated with the named dressings.

Table 3. Chlorhexidine extraction from pork samples covered with wound dressing loaded with different chlorhexidine concentrations

CH[4] concentration in soaking solution (%w/v)	Tissue weight (mg) ±SEM[1]	RT[2] (lido[3])	Area (lido) ±SEM	RT (CH)	Area (CH) ±SEM	CH in sample (ug/ml) ±SEM	Amount of CH in sample (ug) ±SEM	CH in tissue (mg/g)
1	35±6	2.14	15481± 434	3.15	3125±527	20.8±4.27	10.4±2.14	0.32
0.7	41±5	2.14	16321± 724	3.15	2150±425	14.4±3.88	7.2±1.99	0.25
0.4	38±9	2.14	15824± 521	3.14	1125±351	9.9±3.21	4.9±1.62	0.18
0.2	36±4	2.14	16697± 429	3.15	621± 122	5.0±1.75	2.5±0.87	0.12
0.1	40±8	2.14	15482± 821	3.15	215± 136	2.1±1.78	1.0±0.88	0.03

[1] SEM – Standard Error of the Mean [2] RT – Retention time [3] Lido – lidocaine [4] CH – chlorhexidine

3.4 Closing remarks

An efficient and accurate method of chlorhexidine extraction from muscle tissues was developed in the course of this project. The developed method is suitable for extraction of muscle tissue samples of very low weight since its recovery rate is high (>90%). Chlorhexidine concentration in tissue samples covered with wound dressings soaked in various chlorhexidine solutions, was found to be ~10% of the concentration in the

dressing. These results do not assure that the same percentage will be found in the *in vivo* experiment samples because the current *in vitro* experiment does not take factors such as blood circulation into consideration.

The minimal chlorhexidine concentration in the soaking solution (solution in which wound dressings were soaked before applying them to pork samplea) is 0.2%w/v. Lower concentration could not be determined in tissues using HPLC analysis. A more sensitive method of analysis, such as LC-MS, is required to determine lower chlorhexidine concentrations in tissues. Typically, 10 to 50 fold lower concentrations can be accurately measured by LC-MS compared to HPLC.

4.0 STUDY 3: PROCESSING OF EXPERIMENTAL TISSUE SAMPLES: EXTRACTION AND ANALYSIS

4.1 Objective

The objective of this last series of experiments was to determine the chlorhexidine concentration in two sets of pig muscle tissue samples provided by DRDC Toronto, using the extraction procedure developed in Study 2.

4.2 Methodology

The basic extraction procedure previously described in Section 3.0 was slightly modified to make allowance for the small size of the sample, and to allow additional analysis of the samples by LC-MS (steps modified are indicated in bold in Section 4.2.1). Analysis of HPLC results and calculation of chlorhexidine concentration in the tissue samples were performed as described in Section 3.2.2.

4.2.1 Chlorhexidine extraction from pig muscle tissue samples

d) Weigh each sample, and place it into a separate glass tube.

e) Add 20 µl of 1 µg/µl lidocaine solution as an internal standard to each sample.

f) Extract the samples by addition of 5 ml methanol, followed by vigorous vortex shaking and centrifugation (3000 rpm, 10 min, 18°C);

g) Remove the supernatant to a separate glass test tube.

h) Evaporate the supernatant to dryness in a stream of nitrogen at room temperature.

f) **Reconstitute the residue in 400 µl of diluting solvent (water/acetonitrile (65/35)) using a Vortex mixer.**

a) To precipitate impurities, centrifuge the reconstituted sample in ultracentrifuge (3000 rpm, 10 min, 18°C).

b) Draw the sample using a small needle, and filter it through an HPLC syringe-filter (45µm) directly into an HPLC vial.

c) Take 40 µl from the filtered sample and transfer to a clean glass tube.

1. Evaporate 40 µl to dryness in a stream of nitrogen at room temperature.

2. Re constitute the residue in 40 µl of methanol and store in tightly closed glass-tube for LC-MS analysis.

d) Run the filtered samples in HPLC, injecting three times from each sample.

e) Analyse HPLC results as described in Section 3.2.2.

4.3 Results

Tissue weights of all the samples are presented in Tables 4 and 5. These weights have been used for calculation chlorhexidine concentration in the tissues.

Table 4. Tissue weights (March 2002 experiment)

PIG#1	CODE	DAY	WEIGHT, (MG)	PIG#2	CODE		WEIGHT, MG	PIG#3	CODE	DAY	WEIGHT, MG
Blue											
	A1 E	D1	28.0		A1 D	D1	26.2		C1 E	D1	20.8
	B2 B	D1	20.6		C2 E	D1	25.2		A2 C	D1	26.4
	C3 E	D1	5.0		A3 C	D1	21.0		C3 B	D1	9.5
	A4 C	D1	20.0		C4 B	D1	17.5		A4 A	D1	25.9
	B1 A	D1	27.5		B1 B	D1	26.7		D1 A	D1	31.0
	C2 C	D1	14.7		D2 A	D1	18.7		B2 E	D1	9.5
	D3 D	D1	23.7		B3 E	D1	30.2		D3 C	D1	21.9
	D4 B	D1	14.7		D4 C	D1	12.2		B4 B	D1	29.4
Green											
	A1 E	D1	39.8		C2 E	D1	39.6		D3 C	D1	20.8
	B1 A	D1	23.6		D2 A	D1	19.2		A4 A	D1	43.7
	C2 C	D1	25.8		A1 D	D1	36.8		A2 C	D1	16.9
	D3 D	D1	32.5		B1 B	D1	43.2		B4 B	D1	23.8
	A4 C	D1	24.7		C4 B	D1	32.1		D1 A	D1	49.0
	B2 B	D1	52.5		D4 C	D1	45.6		C1 E	D1	31.0
	C3 E	D1	39.7		A3 C	D1	23.9		B2 E	D1	22.2
	D4 B	D1	22.7		B3 E	D1	61.3		C3 B	D1	15.0
Yellow											
	A2 A	D4	30.0		A2 B	D4	22.2		D4 E	D4	19.4
	B3 C	D4	18.0		B2 C	D4	22.8		C4 F	D4	61.9
	D1 C	D4	22.0		D1 E	D4	34.4		A3 E	D4	22.8
	C1 B	D4	19.1		C1 C	D4	36.3		A1 B	D4	40.0
	A3 F	D4	25.2		A4 E	D4	12.5		D2 B	D4	21.1
	B4 E	D4	34.2		B4 A	D4	24.0		C2 D	D4	27.7
	C4 A	D4	39.2		C3 A	D4	26.3		B3 D	D4	42.2
	D2 E	D4	58.4		D3 B	D4	26.5		B1 C	D4	19.5

Table 5. Tissue weights (May 2002 experiment)

14/5/02	BLUE	15/5/02	PINK	17/5/02	YELLOW		YELLOW
CODE	WEIGHT, MG	CODE	WEIGHT, MG	CODE	WEIGHT, MG	CODE	WEIGHT, MG
P1 A1 A	36.3	P1 A1 A	26.6	P1 A1 A	44.9	P2 A1 C	30.9
P1 A4 D	37.1	P1 A2 B	17.4	P1 A2 B	31.0	P2 A2 D	25.0
P1 B2 A	34.7	P1 B1 D	22.6	P1 A3 C	24.1	P2 A3 B	25.0
P1 B3 B	18.3	P1 B2 A	14.0	P1 A4 D	31.0	P2 A4 A	20.7
P1 B4 E	8.2	P1 C1 D	17.8	P1 B1 D	23.8	P2 B1 B	31.0
P1 C2 B	26.4	P1 D4 B	6.0	P1 B2 A	51.9	P2 B2 F	15.0
P1 C4 F	24.0	P1 C3 C	11.6	P1 B3 B	25.1	P2 B3 D	21.9
P1 D1 F	26.0	P1 A3 C	34.7	P1 B4 E	22.8	P2 B4 E	42.0
P1 D2 E	33.7	P2 A1 C	26.7	P1 C1 D	25.9	P2 C1 F	31.4
P1 D3 D	17.1	P2 A2 D	23.2	P1 C2 B	24.3	P2 C2 B	25.0
P2 B1 B	22.7	P2 A4 A	19.6	P1 C3 C	19.2	P2 C3 A	38.2
P2 A4 A	21.5	P2 C2 B	17.0	P1 C4 F	34.6	P2 C4 D	11.5
P2 B2 F	24.6	P2 D1 D	17.4	P1 D1 F	30.0	P2 D1 D	22.5
P2 B3 D	14.8	P2 D2 C	24.7	P1 D2 E	29.6	P2 D2 C	23.7
P2 C1 F	31.7	P2 A3 B	25.0	P1 D3 D	18.3	P2 D3 E	33.6
P2 B4 E	10.4	P2 C3 A	28.5	P1 D4 B	33.1	P2 DR B	29.0
P2 C3 A	10.2						
P2 C4 D	40.8						
P2 D3 E	18.4						
P2 D4 B	18.1						

Tissue samples of two pigs from the March experiment were analyzed. The samples included: Day 1, Day 2 and Day 4 of the experiment. Table 6 summarizes the data. Since no information regarding coding was provided to the Contractor, no conclusions could be drawn from these results.

Table 6. Chlorhexidine concentrations in pig tissues covered with chlorhexidine-loaded DRDC dressings (March 2002 experiment).

Pig	Code	Day	Blue (mg/g)	Green (mg/g)	Pig	Code	Day	Blue (mg/g)	Green (mg/g)
#1	A1 E	D1	0.000	0.000	#2	A1 D	D1	0.007	0.000
	B2 B	D1	0.251	0.008		C2 E	D1	0.010	0.006
	C3 E	D1	0.000	0.000		A3 C	D1	0.332	0.058
	A4 C	D1	0.130	0.048		C4 B	D1	0.099	0.011
	B1 A	D1	0.030	0.010		B1 B	D1	0.168	0.047
	C2 C	D1	0.125	0.024		D2 A	D1	0.025	0.012
	D3 D	D1	0.000	0.000		B3 E	D1	0.000	0.000
	D4 B	D1	0.066	0.015		D4 C	D1	0.085	0.022

4.4 Closing remarks

The current study has implemented the extraction procedure developed in the previous study. The analyzed data show that very low weight samples could be analysed by HPLC using the designed extraction procedure. To confirm HPLC results, a more sensitive method of analysis, such as LC-MS may be used. In order to enable this validation, backups of all extracted samples were prepared for LC-MS analysis. The same extraction procedure was used for preparation of the LC-MS samples.

Since the sample volume for LC-MS analysis is 10-fold lower than that required for HPLC analysis, it is advised that in future experiments, the samples with very low anticipated chlorhexidine concentration would be run exclusively by LC-MS, thus enabling the highest drug concentration in the final sample.

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14. ABSTRACT

(U) The ability of wound dressings to prevent undesired growth of microorganisms when applied to a wound surface is a critical factor in choosing a wound-care strategy. The bactericidal activity of the dressing will not only depend on the type and amount of drug loaded in the material but also on the form in which the drug was loaded. One objective of the current project was to investigate whether the bactericidal activity of the DRDC-Avitar dressing would be prolonged by loading the material with a liposome-encapsulated drug. Various liposomal formulations were tested and the pharmacokinetic profile of each formulation was evaluated. This study revealed that the antibacterial activity of liposomal drug-loaded dressings was prolonged, compared to that of free drug loaded material. However, more extensive studies are required to assess the feasibility of liposome utilisation in wound dressings.
To evaluate the ability of the DRDC-Avitar dressing to deliver the bactericidal drug to the wound, kinetics of drug elimination from the treated tissue as well as the amount of drug remaining in the dressing material after its application to wound must be determined. Since only small tissue samples are obtained from in vivo experiments, the second objective of the project was to develop a drug extraction method that would yield high drug recovery from tested tissues and dressings. The developed procedure has a high recovery rate (>90%) and is easy to perform. The extracted samples may used for HPLC as well as for LC-MS analysis. The validity of HPLC method was confirmed using muscle samples obtained in a previous DRDC experiment.

(U) Quand on soigne une plaie, il est essentiel de choisir un pansement qui empêche des microorganismes indésirables de se multiplier à la surface de la plaie. L'activité bactéricide du pansement ne dépendra pas seulement du type ou de la quantité de médicament incorporé dans le matériel mais aussi de la façon dont le médicament a été incorporé. Le présent projet visait en premier lieu à déterminer si le pansement mis au point par RDDC et Avitar aurait une activité bactéricide plus longue si le médicament incorporé était encapsulé dans des liposomes. Nous avons évalué diverses formulations liposomales dans le cadre de ce projet et nous avons également testé le profil pharmacocinétique de chaque formulation. Les résultats ont révélé que l'activité antibactérienne des pansements à médicament encapsulé dans des liposomes durait plus longtemps que celle des pansements où le médicament incorporé était libre. Des études plus approfondies sont toutefois nécessaires pour juger s'il est faisable d'utiliser les liposomes dans les pansements.

Pour évaluer la capacité du pansement RDDC-Avitar à diffuser le médicament bactéricide dans la plaie, il faut déterminer la cinétique de l'élimination de médicament du tissu traité et la quantité de médicament resté dans le matériel de pansement après l'application de celui-ci. Puisque les expériences in vivo n'ont donné que de petits échantillons de tissus, le second objectif du projet consistait à élaborer une méthode d'extraction de médicament qui permettrait de récupérer des tissus traités et des pansements une bonne quantité de médicament. La procédure, très facile à appliquer, donne un taux élevé de récupération (> 90 %). On peut utiliser les échantillons extraits pour l'analyse par CLHP et par CL/SM.

15. KEYWORDS, DESCRIPTORS or IDENTIFIERS

(U) liposomes; mafenide acetate; HPLC; dressings; chlorhexidine